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Review

Understanding anti-tuberculosis drug efficacy: rethinking bacterial populations and how we model them

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ABSTRACT

Tuberculosis still remains a global health emergency, claiming 1.5 million lives in 2013. The bacterium responsible for this disease, *Mycobacterium tuberculosis* (*M.tb*), has successfully survived within hostile host environments, adapting to immune defence mechanisms, for centuries. This has resulted in a disease that is challenging to treat, requiring lengthy chemotherapy with multi-drug regimens. One explanation for this difficulty in eliminating *M.tb* bacilli *in vivo* is the disparate action of antimicrobials on heterogeneous populations of *M.tb*, where mycobacterial physiological state may influence drug efficacy. In order to develop improved drug combinations that effectively target diverse mycobacterial phenotypes, it is important to understand how such subpopulations of *M.tb* are formed during human infection. We review here the *in vitro* and *in vivo* systems used to model *M.tb* subpopulations that may persist during drug therapy, and offer aspirations for future research in this field.

1. Pathogenesis of *Mycobacterium tuberculosis* – an overview

Tuberculosis, a disease caused by *Mycobacterium tuberculosis*, is primarily transmitted through the respiratory route. Individuals become infected when they inhale aerolised particles produced by patients with active disease. These droplet nuclei (measuring ~1-3 μm and containing 1-3 bacilli) are then engulfed by alveolar macrophages, where *M. tuberculosis* (*M.tb*) bacilli are able to evade killing and continue to multiply by avoiding phagosome-lysosome fusion.[1] Additional macrophages and other immune cells then become localised to the site of infection creating an ordered cellular architecture known as a granuloma. These dynamic structures evolve from simple cellular aggregations with vascular elements to necrotic lesions characterised by hypoxia and nutrient deprivation.[2,3] Caseous necrosis often ensues; this consists of the "solid" necrosis of the exudative lesion and some of the lung tissue that surrounds it. The process likely results in the death of the majority of *M.tb* bacilli but some will survive extracellularly in the solid caseous material. Caseous necrosis can result in the abolishment of the neighbouring host tissues and if this destruction reaches the bronchiolar barrier then a cavity is formed, creating a route of dissemination for *M.tb* bacilli through the airways.[4] The *M.tb* load in tuberculous cavities may reach 10^{11} CFUs (Colony Forming Units) per gram, with bacilli presumably replicating rapidly in this environment.[5] Thus patients with cavitation are highly infectious.[6] Furthermore, the degree of cavitation is often one of the only factors associated with treatment failure.[7] Thus, *M.tb* bacilli are able to survive in multiple diverse and dynamic environments during infection; drug regimens that are able to kill bacilli in all these niches are likely to offer the best opportunity to reduce treatment length and eliminate relapse.

2. *M. tuberculosis* populations within the host

The pathogenesis of *M. tuberculosis* creates bacterial phenotypic heterogeneity, defined here as a mixture of genetically identical bacteria that vary in measured characteristic(s). This heterogeneity may impact upon the metabolic state of *M.tb* and/or the efficacy of antimicrobials. Thus *M.tb* infection rather resembles, and might be approached as, a polymicrobial infection where several cidal activities are required for *M.tb* sterilisation by chemotherapy.

When exposed to bactericidal concentrations of antimicrobial drugs, the number of viable cells in a susceptible bacterial population does not decline exponentially. Instead, the mortality rate decreases over time and a substantial fraction of the population may survive antimicrobial drug treatment. This

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phenomenon has been observed for virtually all antimicrobials used in clinical practice and for many bacterial species[8–14] and has been attributed to antimicrobial tolerance. Phenotypic antimicrobial tolerance is a temporary, reversible bacterial state that is often associated with a reduced rate of multiplication, where some antimicrobial drugs are ineffective against genetically susceptible bacilli. Antimicrobial tolerance is hypothesised to be the prime reason for the extended treatment regimens required for *M.tb* chemotherapy, as fully drug-sensitive bacilli survive (persist through) initial antimicrobial drug therapy.[15–17] It has long been speculated that *M.tb* in a non/slowly-replicating state may play a clinically significant role, persisting during drug therapy.[18–20] The first-line antimicrobials used to treat *M.tb* infection (isoniazid, rifampicin, pyrazinamide and ethambutol) are all active against actively-replicating bacteria,[21] however effectiveness of this drug regimen against non/slowly-replicating bacilli is reduced or eliminated.[22–24] This *M.tb* slow/non-replicating state is hypothesised to be induced by the environmental conditions found in specific granuloma types, in particular those associated with hypoxia or nitric oxide production.[18,25,26] An *M.tb* transcriptional signature resembling slow or non-replicating bacilli was also identified in bacilli isolated from human sputa.[27] Exposure of bacilli to such microenvironments results in the expression of a discrete set of genes known as the dormancy regulon (DosR/DevR) that are in turn responsible for transitioning bacilli into a non-replicating and hence likely drug-tolerant state.[25,28-30] The mechanism(s) that result in the generation of phenotypic drug tolerant *M.tb* populations *in vivo* are currently not well understood, however it is critical to consider these sub-populations of bacilli for the development of more effective drug regimes.

The *M.tb* population *in vivo* has been compared to a Russian nesting doll, consisting of layer after layer of distinct bacterial subpopulations that may also be separated by time and space, each of which may be differentially killed by various antimicrobial drugs dependent on phenotypic drug tolerance or anatomical location. The models developed by Mitchison[31] and Mitchison and Coates[17,32] are often adapted to describe four populations defined by antimicrobial drug efficacy (1) actively growing bacilli mostly killed by isoniazid, (2) slow/non-replicating *M.tb* bacteria that undergo spurts of metabolism, which are killed by rifampicin, (3) intracellular bacilli present in the acidic compartments of macrophages or in acidic lung lesions that are killed by pyrazinamide, and (4) *M.tb* persisters found in hypoxic microenvironments with much reduced action of most anti-TB drugs.[22,33] As evidenced in TB patients that relapse during treatment of drug-sensitive *M.tb*, the host immune system cannot effectively eliminate these residual *M.tb* bacilli that are not killed by chemotherapy. Therefore, although achieving a clinical cure, the current anti-TB standard regimen does not necessarily achieve a bacteriological cure. In other words, current therapy does not completely eradicate all bacilli from the body, but allows the infection to be contained effectively for long-periods of time.[34] These observations underscore the need for developing better sterilising compounds against *M.tb*. However, the lack of adequate screening systems able to identify new compounds effective against drug-tolerant *M.tb* phenotypes remains an immense barrier to the anti-tuberculosis drug development process.[35]

3. Models to study *M. tuberculosis* populations that may persist during drug therapy

3.1. In vitro studies

Several models have been developed to recreate conditions encountered by *M.tb* within the host during infection. Since the nomenclature surrounding *M.tb* dormancy and latency is ambiguous, we refer to these models here simply as systems that generate populations of bacilli that may persist (or at least be differentially killed) by antimicrobials during disease. The *in vitro* systems necessarily only capture specific aspects of the clinical scenario. These models mimic stimuli hypothesised to be present during infection and may be divided into two groups: (1) Those designed to generate a largely homogenous bacterial population to characterise specific responses and develop drug screens for defined bacterial phenotypic states. (2) Those aimed at producing mixed populations of *M.tb*, often with multiple stimuli to model drug action on heterogeneous populations. Of course, bacterial heterogeneity is entirely defined by the methods used to characterise *M.tb* populations and the resolution of the techniques.

The method described by Wayne and colleagues is the most frequently used experimental approach to hypoxia and, hence, the best characterised. In this model, *M.tb* is grown under agitation in air-tight containers with a defined headspace-to-culture ratio and for a defined period of time (usually 24 days), which leads to the gradual depletion of oxygen.[23] When deprived of oxygen, the bacilli enter a non-replicative state that is refractory to isoniazid-dependent killing.[36] This physiological state may be reversed by exposure to atmospheric oxygen conditions.[22,37] The Wayne model has been used for the evaluation of new compounds; non-replicating phase 2 (NRP2) bacilli were treated with test drugs and subsequent mycobacterial growth was determined by conventional plating methods.[38] Using similar methodology, metronidazole and PA-824 (a nitroimidazo-oxazine now in phase 2 and 3 clinical trials) were shown to be active against anaerobic *M.tb in vitro*.[38] Several versions of the Wayne model have been developed to increase its throughput capacity, for example, by combining with colorimetric or luminescence-based measures of bacterial viability.[35,39,40] In addition, using multiple genome-wide analyses, Galagan and colleagues have been able to explore the molecular mechanisms that are employed by *M.tb* during hypoxia and reaeration phases.[41]

Deprivation of nutrients is another stress hypothesised to be encountered by *M.tb* inside granulomas. The models that reproduce this condition normally involve the incubation of tubercle bacilli in minimal medium for approximately 6 weeks. During this period, the cells undergo a global metabolic shift:[42,43] several metabolic pathways are shut down and lipids become the sole source of energy;[44] while rescue pathways, such as those involved in the synthesis of vital enzymes, are upregulated. Aerobic respiration usually shuts down after ~9-12 days. Starvation-induced persistent-bacilli are tolerant to some antimicrobials, such as isoniazid, rifampicin and metronidazole. However, pyrazinamide, econazole and clotrimazole are active against this *M.tb* population.[42,45,46] Chemostat models are a key resource for such investigations where a controlled environment is achieved by fine-tuning bacterial growth rate alongside parameters affecting the respiratory and metabolic state of bacilli. Chemostats have been successfully used to study the molecular adaptations of mycobacteria to nutrient depletion,[47] low oxygen tension,[48] and between fast-growing and slow-growing populations.[49] Additional single stress models have been developed by either limiting the availability of specific nutrients or inducing a stress predicted to be present *in vivo*, for example low potassium,[50] PBS starvation model,[42] reduced oxygen, low pH.[51,52]

Hypoxia and nutrient starvation models can trigger *M.tb* to slow growth and activate the DosR/DevR regulon, however they cannot simulate the multiple environmental stimuli that are likely found within granulomas, as bacilli adapt to the dynamic surroundings. For this reason, multiple-stress models (combining hypoxia, nutrient starvation, low pH) may offer a more complete *in vitro* simulation of the circumstances bacilli encounter in some human lung lesions.[53] The model developed by Deb *et al.* was shown to

produce non-replicating cells that possess all the hallmark characteristics of bacilli that may persist during drug therapy. This model has not been used widely for drug screening but might represent a good alternative. In addition, *in vitro* biofilm models have been developed to model the generation of complex mycobacterial populations, and antimicrobial drug-tolerant states, which may be utilised toward exploring the action of novel drugs against heterogeneous *M.tb* populations.[54] Furthermore, nanotechnology systems such as microfluidic devices offer revolutionary novel approaches to studying bacterial subpopulations and the development of persisting organisms by monitoring the fate of single bacterial cells within a population.[55,56]

All the above *in vitro* models have the obvious limitation of not being able to reproduce the effects of the host: immune response, cellular architecture, macrophage phagocytosis and eventual release to the extracellular milieu. Macrophage infection models are available that mimic the early *M.tb* responses to the host immune system but fail to capture long-term metabolic changes.[25,57-59] The transcriptional adaptations of *M.tb* to the intracellular environment have also been defined using mRNA profiling to identify key metabolic and respiratory changes, and to identify factors that drive the generation of *M.tb* phenotypes during infection.[60-63] Intracellular models of drug action reveal that antimicrobials differ in their abilities to kill intracellular bacteria, likely a combination of drug penetration and efficacy against intracellular-adapted *M.tb* phenotypes that may be in part drug-tolerant.[58] Techniques such as high content screening have been developed to model intracellular drug efficacy, alongside drug penetration and toxicity.[59]

Another possibility is the development of *in vitro* human granuloma models.[64] The availability of a granuloma model could provide a useful strategy for the study of drug efficacy against drug-tolerant *M.tb*. Several groups have attempted to develop such a model,[65-67] but, thus far, only one has generated *M.tb* cells with the characteristics of persistent mycobacteria.[68]

3.2. Pre-clinical animal models

There are several animal models available for the study of persistent TB infection and the determination of the *in vivo* activity of novel compounds; these include mouse, guinea pig, rabbit, non-human primate and zebrafish models.[69] Animal models not only offer the possibility of circumventing the limitations discussed above (by including host in the modelling) but also allow an early assessment of the drug's toxicity to the host, and the ability to test a compound's activity against cell processes that only manifest *in vivo* during host/pathogen interaction.[70] However, the difficulties in developing and manipulating animal models of TB infection, and the assessment of the clinical relevance of each system, means that animal models are also not without limitations.

There are two established murine models for the study of latent TB, which generate populations of *M.tb* that persist through drug treatment. (1) In the Cornell model[69] mice are inoculated with high dose ($1x10^6$-$3x10^6$ CFU) *M.tb* bacilli and subsequently treated with isoniazid and pyrazinamide for 12 weeks. By the end of this treatment, the mice do not show obvious signs of disease and the bacterial loads in organ homogenates are reduced to undetectable levels, suggested to mimic latent human infection. The presence of *M.tb* populations that persist through drug therapy is shown by spontaneous (in about one third of animals by week 3 after treatment) or steroid-induced (in almost all mice) relapse. The bacilli recovered from these murine relapse cases are acid fast and fully susceptible to isoniazid and pyrazinamide administered before. Several modified versions of this model optimising parameters such as duration of drug therapy and drug dosages have been proposed.[71,72] (2) In the second model, mice are inoculated via the respiratory route with a low dose of *M. tuberculosis* (5-10 CFU).[73] This low dose model relies solely on the natural host immune response to control the infection; approximately 3 months after inoculation the pulmonary bacterial load stabilises at around ~3 to 4 $\log_{10}$.[73] This clinically quiescent phase of the infection may be maintained for up to 1.5 years, after which the mice begin to relapse. To determine the activity of new compounds against *M.tb* in these models, test drugs are administered to mice in the latent phase of infection, followed by immunosuppressive treatment to allow the reactivation of bacilli and enumeration of viable cells in tissue homogenates.[74] Using this approach, metronidazole was found to be unable to prevent reactivation of TB infection in mice, while the azole antifungal econazole was shown to significantly reduce the bacterial burden in lungs and spleens of these infected mice.[46,75] However, the disparate TB pathologies between murine and human lungs, mice do not develop organised granulomas with caesous necrosis or mineralisation,[45,76] mean that these observations may not be reproduced in human studies.

The gross pathology of tuberculosis disease in guinea pigs and rabbits more closely resembles that of humans, with similar mechanisms of granuloma formation and associated caseation.[77-80]. Furthermore, rabbit granulomas may progress to liquefaction and cavitation.[81,82] The rabbit model of latent TB[83,84] is characterised by persistent, host-contained paucibacillary infection that may be reactivated by immunosuppressive treatment. This makes it an attractive model to study drug efficacy against persistent *M.tb*.[2] In addition, the existence of non-replicating tubercle bacilli in lung lesions of guinea pigs, sharing similarities with those found in humans, has been confirmed.[79] The similarities between *M.tb* natural infection in humans and guinea pigs also provide evidence to suggest that non-human primate models are probably the most relevant for studying drug-persistent TB as they capture the spectrum of human tuberculous lung pathology.[25,69] However, these are also the most costly and resource-intensive models.[2] This, together with concerns over the reproducibility of these models (associated with genotype variability and small sample sizes) and adverse public opinion, limit the widespread use of non-human primate models for TB drug discovery programmes.[69,85]

4. Need for better models to evaluate the efficacy of drugs against specific M.tb subpopulations

Human infection with *M.tb* is a complex and multi-factorial process involving the immuno-modulation and remodelling of multiple tissues over many years. Within granulomas, for example, there is a gradient of active, inactive and dead immune cells and bacteria, changing oxygen potential and nutrient composition. These diverse scenarios result in a complex *M.tb* population structure, composed of bacilli in different metabolic states that are killed at different rates by the spectrum of antimicrobial drugs.

The requirement for lengthy TB chemotherapy likely reflects the inability of current antimicrobial drugs to eradicate subpopulations of *M.tb* bacilli, enabling these populations of bacteria to persist in infected individuals. It is therefore crucial to develop new drugs that can effectively eliminate these persisting cells. A sequence of *in vitro* assays and animal models of *M.tb* mimicking TB infection are applied in drug discovery and development to identify and select active compounds. Unfortunately, the tools currently available have a limited ability to predict the activity of new drugs against *M.tb* bacilli that are able to survive in human disease during chemotherapy. It is therefore critical to develop practical, robust, and high-throughput models that can accurately reproduce *M.tb* subpopulations found *in vivo*; this will enable novel

regimens to be selected that target key *M.tb* populations to affect sterilisation more effectively, reducing treatment length.

Persistence through drug therapy in *M.tb* has been associated with a non-replicative state resistant to standard anti-TB drugs (such as isoniazid and rifampicin), loss of acid-fastness, RPF-dependency and accumulation of lipid bodies. Many *in vitro* models are able to induce a non-replicative *M.tb* state but fail to demonstrate or measure other characteristics. Thus, it is currently unclear how the phenotype of sub-populations of bacilli from different models overlap, and how important each stimulus is to the generation of bacteria that may survive prolonged chemotherapy.

Murine models are most frequently used for determining the sterilising activity of new compounds, however the translation of these results to the human system is problematic since tuberculous mice lack lesion types characteristic of human disease. Re-engineering the host (and/or pathogen) may succeed in transforming TB disease immunopathology to more closely resemble that of human infection. An alternative approach sees the adoption of a number of different models alongside the murine model that reflect the progression of human lung pathology for the study of drug efficacy in lung lesions. Either way, the development of better strategies for drug screening is hampered by the lack of adequate knowledge about the biological features of both the tubercle bacilli and host immunity during TB infection of the human lung. We suggest the following priorities for future research into the significance of *M.tb* population heterogeneity in human disease and novel drug development strategies:

1. We need better models that define the action of anti-TB drugs against specific sub-populations of *M.tb* bacilli, characterised using standardised parameters that may be used to compare between systems.
2. We require methodologies for characterising complex populations of bacilli, in an alternative approach to (1.), to directly mimic the heterogeneous *M.tb* population structure found during human disease.
3. Definitions of bacterial states (for example: dormant, latent, active growth, tolerant) would be useful to align thinking and enable study comparison.
4. Further exploration is needed into the role of host processes in generating sub-populations of phenotypically-distinct bacilli and knowledge of whether these subsets of bacilli are confined to specific anatomical locations during disease.
5. Finally, an understanding of the clinical significance of these *M.tb* sub-populations in human disease is necessary; does population size or location matter, and is there significant patient-to-patient variation?

This is a tall order, and successful anti-TB drug regimens have been developed in the past with little knowledge of these processes. However, a greater understanding of the action of existing and novel antimicrobials on *M.tb* subpopulations that are able to persist through drug therapy will drive a paradigm shift in TB chemotherapy options, significantly reducing treatment length and eliminating relapse.

Conflict of Interest

None

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